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ON THE ADMINISTRATION OF DIPHTHERIA TOXIN IN A COLLODION SAC.*

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The therapeutic value of antidiphtheric serum is recognized by all who are conversant with the facts; as to how it acts and how its value can best be tested there is much less unanimity. The Germans, following Behring and Ehrlich, stand for the antitoxic unit as the full measure of therapeutic value, while the French, following Roux, contend that the antitoxic power of a serum is not a satisfactory measure of its therapeutic value. The reason why this question cannot be definitely settled is because diphtheria, as found in man, cannot be produced experimentally in animals. Hence the curative value of a serum can be tested only on human beings and these of course cannot be adequately controlled. On the other hand, the antitoxic value of a serum can be easily and accurately tested on guinea-pigs and this accounts for the universal use of the antitoxin unit. The question still remains: Does the antitoxic power of a serum adequately measure its curative power?

In the attempts which have been made to devise a satisfactory test for the therapeutic value of antidiphtheric serum the use of diphtheria cultures either on scarified mucous membranes, or by subcutaneous injection, has been abandoned because of irregular results, and recourse has usually been had to the subcutaneous injection of toxin followed after an interval by the injection of antitoxin. This method differs from the conditions found in man, chiefly as to the rapidity with which the toxin is absorbed. In man the toxin is absorbed from the false membrane at first slowly, later more rapidly, until some influence occurs which checks the production of toxin. When the toxin is injected subcutaneously it is absorbed rapidly and the system is suddenly overwhelmed with toxin. In casting about for some method of introducing toxin into animals more analogous to the manner of its absorp-

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tion from the false membrane in man, recourse was had to a collodion sac containing toxin, placed in the peritoneal cavity of a guinea-pig. It was hoped that the toxin would be absorbed slowly from the sac and produce death in a manner analogous to a case of diphtheria in man. After much preliminary work on the size and quality of sac and amount of toxin, a combination was found which would uniformly kill 400 gm. guinea-pigs in five, six, or seven days after the introduction of the sac.

Histological examination of the abdominal organs showed no lesions other than those usually found in diphtheria, hence we concluded that death was not due to any local action of the toxin but to the usual systemic action and that absorption from the sac corresponded quite well to absorption from the false membrane. In all cases careful bacteriological tests were made of the peritoneal fluid, the cultures being both aerobic and anaerobic and the media in all cases containing unheated animal proteid such as ascitic fluid, blood serum, liver extract, etc. In most cases the peritoneum was entirely sterile. The few cases in which infection occurred are not included.

TABLE I.

DETERMINATION OF L+ DOSE OF DIPHTHERIA TOXIN NO. 05656.
L+ DOSE TAKEN AS 0.27 C.C.

GUINEA-PIG	SERUM		TOXIN		REACTION TIME IN MINUTES	INJECTION	DEATH	INTERVAL IN DAYS
Weight	Name	Dose	℞ No.	Dose		Day	Day	
250	Ehrlich	1 unit	05656	0.25	20	9–16–09	9–24–09	8
"	"	"	"	.25	"	"	9–22–09	6
"	"	"	"	.26	"	"	9–22–09	6
"	"	"	"	.26	"	"	9–21–09	5
"	"	"	"	.27	"	9–21–09	9–25–09	4
"	"	"	"	.27	"	"	9–25–09	4
"	"	"	"	.27	"	"	9–24–09	3

The results here given are the outcome of this work with collodion sacs and altho the work is by no means finished, yet it seems of sufficient interest to report. It is the hope of the writer, who is unable to continue the work, that someone else may take it up for further investigation.

Preliminary Tests.—The M.L.D. and L+ dose of a test toxin were carefully worked out as shown in Tables 1 and 2, and a quantity of this toxin was put up in small vials for use in this work. The antitoxic potency of two sera was determined; one being of relatively high potency (900 units per c.c.) and one of relatively low potency (200 units per c.c.) as shown in Table 3. Quantities of these sera were set aside.

Considerable time was consumed in determining the size and quality of sac and the amount of toxin required to give the desired results. The ultimate results, only, of this phase of the work need be given. We found that a sac having a capacity of about 1.5 c.c. and containing 0.15 c.c. of toxin (about 37½ M.L. doses or 55 per cent of the L+ dose) would kill a 400 gm. guinea-pig in five, six, or seven days as shown in Table 4.

TABLE 2.

DETERMINATION OF THE MINIMUM LETHAL DOSE OF DIPHTHÈRIA TOXIN NO. 05656
M.L. DOSE TAKEN AS 0.004.

GUINEA-PIG	TOXIN		INJECTION	DEATH	INTERVAL IN DAYS	PERCENTAGE OF DEATHS
Weight	℞ No.	Dose	Day	Day		
250	05656	0.0032	9-28-09	....	..	43
“	“	“	11-10-09	11-29-09	9	
“	“	“	12- 2-09	12- 6-09	4	
“	“	“	12- 7-09	....	..	
“	“	“	12-15-09	....	..	
“	“	“	12-28-09	1- 3-10	6	
“	“	“	1-15-10	1-21-10	6	
“	“	0.0034	10- 5-09	10-10-09	5	62½
“	“	“	10-11-09	10-18-09	7	
“	“	“	11-10-09	11-16-09	6	
“	“	“	12- 2-09	12- 6-09	4	
“	“	“	12- 7-09	....	..	
“	“	“	12-15-09	12-22-09	7	
“	“	“	12-28-09	....	..	
“	“	“	1-15-10	....	..	
“	“	0.0036	10- 5-09	....	..	71½
“	“	“	11-10-09	11-16-09	6	
“	“	“	12- 2-09	12- 6-09	4	
“	“	“	12- 7-09	12-11-09	4	
“	“	“	12-15-09	12-22-09	7	
“	“	“	12-28-09	1- 2-10	5	
“	“	“	1-15-10	....	..	
“	“	0.0038	10- 5-09	10-10-09	5	80
“	“	“	10-11-09	10-18-09	7	
“	“	“	10-21-09	....	..	
“	“	“	11-18-09	11-26-09	8	
“	“	“	11-10-09	11-13-09	3	
“	“	0.0040	10- 5-09	10-11-09	6	100
“	“	“	10-11-09	10-15-09	4	
“	“	“	10-21-09	10-28-09	7	
“	“	“	10-28-09	11- 1-09	4	
“	“	“	11- 4-09	11- 8-09	4	
“	“	“	11-10-09	11-16-09	6	
“	“	“	11-18-09	11-29-09	11	

This secured a satisfactory interval and the dose (0.15 c.c.) has been adhered to since. It was early found that the collodion sacs must be uniform in thickness and quality or the results would be irregular. After considerable experimenting a method of making them was developed, which has given satisfactory results and has since been adhered to.

The method is a modification of that given by Frost.[1] Nine parts of Schering's celloidin are added to 100 parts of a mixture of equal parts of absolute alcohol and ether in a bottle. The mixture is allowed to stand with occasional shaking for about 24 hours, then placed in a mechanical shaker and shaken until dissolved. After all air bubbles have disappeared, a portion is poured into a test-tube (size 12×100 mm.), the tube is rotated, and the excess poured out into another bottle, not back into the unused collodion. The tube is rotated in a horizontal position for about $\frac{1}{2}$ a minute to distribute the adhering collodion uniformly, then it is placed in a vertical position with the open end down and allowed to drain for 70 seconds. The excess of collodion about the lip of the tube is now removed and the tube is placed in a vertical position with the mouth up for $2\frac{1}{2}$ minutes. A capillary glass tube having an internal diameter of 1 mm. is now introduced into the test-tube nearly to the bottom, and air under $\frac{3}{4}$ of a pound pressure per square inch is allowed to blow through the capillary for three minutes. The tube is now filled with water and allowed to stand till the next morning, when the tube of collodion can easily be withdrawn from the glass tube. The collodion tubes are suspended in test-tubes of water by means of a thread and sterilized. After some practice, it is possible to make very perfect tubes by this method. When wanted for use a tube is withdrawn by means of the string from the test-tube in which it was sterilized and placed in a short sterile test-tube (about 12×25 mm.) held in the hand. The top can now be cut off where desired, the water withdrawn with a pipette, and the desired amount of toxin and water introduced. The tube is then closed by tying the string, and sealed with a drop of collodion. The sac thus prepared is introduced into the peritoneal cavity of a 400 gm. guinea-pig, strictly aseptic precautions being observed during the operation.

TABLE 3.

POTENCY TEST OF TWO ANTIDIPHTHERIC SERUMS, NO. 013069 TESTING 900 UNITS PER C.C. AND NO. 013327 TESTING 200 UNITS PER C.C.

GUINEA-PIG	SERUM		TOXIN		REACTION TIME IN MINUTES	INJECTION		DEATH	INTERVAL IN DAYS	RELEASE
Weight	Name	Dose	℞ No.	Dose		Day	Hour	Day		
250	013069	0.00117	05656	0.27	20	9–24–09	4 P.M.			9–30–09
“	“	.00111	“	“	“	“	“			9–30–09
“	“	.00105	“	“	“	“	“	9–27–09	3	
“	013327	.0050	“	“	“	“	“			9–30–09
“	“	.0040	“	“	“	“	“	9–28–09	4	
“	“	.0033	“	“	“	“	“	9–27–09	3	

Curative Tests.—Having found a method by which guinea-pigs could be killed by the gradual absorption of diphtheria toxin from a collodion sac, antitoxin was administered to determine its value in saving the lives of those guinea-pigs into which such sacs had been inserted.

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[1] *Laboratory Guide to Bacteriology*, p. 296. Madison, Wis., 1902.

In all these tests the same amount of toxin was placed in the sacs, namely, 37½ M.L.D. or as much as would be neutralized by 0.55 of a unit. In Table 5 the results are shown when the serum is administered in one dose by subcutaneous injection 24 hours after the introduction of the sac. It will be seen that the animals receiving antitoxin survive longer than the controls, the length

TABLE 4.

CONTROL GUINEA-PIGS SHOWING TIME OF DEATH AFTER THE INTRODUCTION OF A COLLODION SAC CONTAINING 37½ FATAL DOSES OF DIPHTHERIA TOXIN NO. 05656.

The average time of death of the 41 guinea-pigs in this series is almost exactly six days.

GUINEA-PIG	TOXIN IN SAC		INTRODUCTION OF SAC	DEATH	INTERVAL IN DAYS
Weight	℞ No.	Dose	Day	Day	
400	05656	0.15	9- 7-09	9-13-09	6
“	“	“	9-13-09	9-18-09	5
“	“	“	9-14-09	9-20-09	6
“	“	“	9-20-09	9-27-09	7
“	“	“	9-20-09	9-27-09	7
“	“	“	9-20-09	9-27-09	7
“	“	“	10- 1-09	10- 6-09	5
“	“	“	10- 6-09	10-12-09	6
“	“	“	10-12-09	10-18-09	6
“	“	“	10-19-09	10-24-09	5
“	“	“	10-18-09	11- 3-09	6
“	“	“	12-14-09	12-21-09	7
“	“	“	12-14-09	12-19-09	5
“	“	“	12-14-09	12-20-09	6
“	“	“	12-21-09	12-26-09	5
“	“	“	12-21-09	12-26-09	5
“	“	“	12-21-09	12-27-09	6
“	“	“	12-29-09	1- 5-10	7
“	“	“	12-29-09	1- 4-10	6
“	“	“	1-25-10	2- 1-10	7
“	“	“	1-25-10	1-31-10	6
“	“	“	1-25-10	1-31-10	6
“	“	“	2- 7-10	2-13-10	6
“	“	“	2-23-10	3- 2-10	7
“	“	“	2-23-10	3- 1-10	6
“	“	“	2-23-10	3- 1-10	6
“	“	“	3- 9-10	3-15-10	6
“	“	“	3- 9-10	3-15-10	6
“	“	“	3-23-10	3-29-10	6
“	“	“	4- 2-10	4- 7-10	5
“	“	“	4- 2-10	4- 7-10	5
“	“	“	4- 2-10	4- 8-10	6
“	“	“	4-11-10	4-16-10	5
“	“	“	4-11-10	4-16-10	5
“	“	“	4-11-10	4-17-10	6
“	“	“	4-11-10	4-17-10	6
“	“	“	4-13-10	4-20-10	7
“	“	“	4-29-10	5- 4-10	5
“	“	“	5- 7-10	5-12-10	5
“	“	“	6- 3-10	6-10-10	7
“	“	“	6-14-10	6-21-10	7

of time they survive being roughly proportional to the amount of antitoxin they receive up to a point beyond which they do not die. However, the results are very irregular, especially in relation to the amount of antitoxin necessary to really save the life of the guinea-pig. It seems absurd that it should require more than 80 units to save the life of the pig, when the entire amount of toxin originally placed in the sac would be neutralized *in vitro* by one-half of a unit. The apparent explanation of the large amount of antitoxin required seemed to lie in the destruction of antitoxin by the tissues of the guinea-pig. Accordingly the method of administering the antitoxin was changed from one dose 24 hours after the introduction of the sac to four doses—one on the day of the operation and one on each of the three following days. The results are shown in Table 6.

TABLE 5.

SHOWING THE RESULTS OF INJECTING ONE DOSE OF ANTITOXIN VARYING FROM 1 TO 1,280 UNITS, 24 HOURS AFTER THE INTRODUCTION OF A SAC CONTAINING 37½ FATAL DOSES OF TOXIN.

GUINEA-PIG	SERUM		TOXIN IN SAC		INTERVAL IN HOURS	INTRODUCTION OF SAC	DEATH	INTERVAL IN DAYS	AV. INTERVAL
Weight	℞ No.	Dose	℞ No.	Dose		Day	Day		
400	013069	1 unit	05656	0.15	24	10- 6-09	10-12-09	6	7.75 days
“	“	“	“	“	“	10-12-09	10-21-09	9	
“	“	“	“	“	“	10-13-09	10-20-09	7	
“	“	“	“	“	“	10-28-09	11- 6-09	9	
“	“	2 units	“	“	“	10- 6-09	10-14-09	8	10 days
“	“	“	“	“	“	10-12-09	10-22-09	10	
“	“	“	“	“	“	10-13-09	10-22-09	9	
“	“	“	“	“	“	10-28-09	11-10-09	13	
“	“	5 units	“	“	“	10-12-09	10-25-09	13	13.3 days
“	“	“	“	“	“	10-13-09	10-21-09	18	
“	“	“	“	“	“	10-28-09	11-16-09	19	
“	“	10 units	“	“	“	9-28-09	10-13-09	15	14 days
“	“	“	“	“	“	10- 1-09	10-14-09	13	
“	“	20 units	“	“	“	10- 1-09	10-19-09	8	15.5 days
“	“	“	“	“	“	2- 3-10	2-16-10	13	
“	“	40 units	“	“	“	10- 1-09	10-14-09	13	19.5+ days
“	“	“	“	“	“	12-23-09	1-18-10	26	
“	“	“	“	“	“	2- 3-10	....	..	
“	“	“	“	“	“	2-15-10	....	..	
“	“	“	“	“	“	4-29-10	....	..	
“	“	80 units	“	“	“	12-23-10	1- 9-10	17	
“	“	“	“	“	“	2- 3-10	2-23-10	20	
“	“	“	“	“	“	2-15-10	3- 3-10	16	
“	“	“	“	“	“	4-29-10	....	..	
“	“	160 units	“	“	“	12-23-09	....	..	
“	“	320 units	“	“	“	12-23-09	....	..	
“	“	640 units	“	“	“	12-23-09	....	..	
“	“	1280 units	“	“	“	12-23-09	....	..	

It will be seen that when given in four doses on successive days following the introduction of the sac about as large doses are required as when given in a single dose; four doses of 20 units each being required to save the guinea-pig. In each case the animal survives as long as the antitoxin remains in its system. When the antitoxin is exhausted the toxin still diffusing from the sac kills the guinea-pig.

TABLE 6.

ANTITOXIN ADMINISTERED TO A SERIES OF FIVE GUINEA-PIGS IN FOUR SUCCESSIVE DOSES AT INTERVALS OF 24 HOURS FOLLOWING THE INTRODUCTION OF A COLLODION SAC.

Four doses of 20 units each were required to save the guinea-pig.

GUINEA-PIG	SERUM SUBCUTANEOUSLY		TOXIN IN SAC		INJECTION		DEATH	INTERVAL IN DAYS	RELEASE
Weight	℞ No.	Dose	℞ No.	Dose	Day	Hour	Day		
400	......	......	05656	0.15	2–16–10	3 P.M.			
"	013327	5 units	......	....	2–16–10	5 P.M.			
"	"	"	......	....	2–17–10				
"	"	"	......	....	2–18–10				
"	"	"	......	....	2–19–10		3–1–10	13	
400	......	....	05656	0.15	2–16–10	3 P.M.			
"	013327	10 units	......	....	2–16–10	5 P.M.			
"	"	"	......	....	2–17–10				
"	"	"	......	....	2–18–10				
"	"	"	......	....	2–19–10		3–6–10	18	
400	......	....	05656	0.15	2–16–10	3 P.M.			
"	013327	20 units	......	....	2–16–10	5 P.M.			
"	"	"	......	....	2–17–10				
"	"	"	......	....	2–18–10				
"	"	"	......	....	2–19–10				3–30–10
400	......	....	05656	0.15	2–16–10	3 P.M.			
"	013327	40 units	......	....	2–16–10	5 P.M.			
"	"	"	......	....	2–17–10				
"	"	"	......	....	2–18–10				
"	"	"	......	....	2–19–10				3–30–10
400	......	....	05656	0.15	2–16–10	3 P.M.			
"	013327	80 units	......	....	2–16–10	5 P.M.			
"	"	"	......	....	2–17–10				
"	"	"	......	....	2–18–10				
"	"	"	......	....	2–19–10				3–30–10

That the supply of antitoxin might continue as long as the supply of toxin, another series was started in which the serum was injected every third day, beginning with the day after the operation and continuing until the animal died or it was seen that there was no longer probability that it would die. Table 7 gives details and results. As will be seen, 1, 2, 4, and 8 units given every third day do not save the animal but prolong its life to 10, 13, 15, and 31 days respectively, while 16 and 32 units every third day protect the animal completely. Another series was run in which the antitoxin was injected daily, beginning with the day of the operation and continuing till the animal died or the danger of death was past. Table 8 gives the details. In this case doses running from 0.1 to 2.0 units injected daily prolonged the life of the animal but failed to save it, while 4 units daily saved it. As a final step an attempt was made to administer the antitoxin as continuously as the toxin by allowing it also to diffuse from collodion sacs placed in the peritoneal cavity; both sacs being introduced at one operation. That there might be more surface for diffusion two sacs containing serum were used and the results are shown in Table 9. The results would indicate that there was some diffusion of antitoxin through the sacs, as those animals in which the sacs contained a large number of units did not die. However, attempts to obtain diffusion of antitoxin through similar sacs *in vitro* failed completely.

TABLE 7.

ANTITOXIN ADMINISTERED EVERY THIRD DAY TO GUINEA-PIGS CARRYING SACS OF DIPHTHERIA TOXIN.

Sixteen units every third day saved the animal; eight units did not.

SERUM SUBCUTANEOUSLY		TOXIN IN SAC		INJECTION	DEATH	INTERVAL IN DAYS
℞ No.	Dose	℞ No.	Dose	Day	Day	
......	....	05656	0.15	2– 2–10		
013069	1 unit	.....	....	2– 3–10		
"	"	every third day up to		2– 9–10	2–12–10	10
......	....	05656	0.15	2– 2–10		
013069	2 units	.....	....	2– 3–10		
"	"	every third day up to		2–12–10	2–15–10	13
......	....	05656	0.15	2– 2–10		
013069	4 units	.....	....	2– 3–10		
"	"	every third day up to		2–15–10	2–17–10	15
......	....	05656	0.15	2– 2–10		
013067	8 units	.....	....	2– 3–10		
"	"	every third day up to		3– 2–10	3– 5–10	31
......	....	05656	0.15	2– 2–10		
013069	16 units	.....	....	2– 3–10		
"	"	every third day up to		3– 2–10	Released 3–30–10 in good condition.	
......	....	05656	0.15	2– 2–10		
013069	32 units	.....	....	2– 3–10		
"	"	every third day up to		3– 2–10	Released 3–30–10 in good condition.	

These curative tests emphasize the important part played by the destruction of antitoxin *in vivo* by showing clearly that a small amount of toxin protected from destruction by being inclosed in a sac and slowly diffusing out into the peritoneal cavity is effective against relatively large doses of antitoxin injected into the subcutaneous tissue where they are exposed to rapid destruction.

They also emphasize the difference between this work with the sac and true diphtheria by showing that eight units every third day do not protect the guinea-pig from the continuous administration of small quantities of toxin. Eight units for a 400 gram guinea-pig correspond to 1,345 units for a man, and this quantity every third day would certainly protect a man from infection and probably cure most cases. Now the reason this quantity does not protect the guinea-pig is, in all probability, because the absorption of toxin is continuous irrespective of whether it be neutralized or not, while a corresponding quantity saves a man not alone because it neutralizes the toxin already absorbed but because it stops the supply of toxin. We know clinically that following an injection of serum the false membrane changes in appearance and comes off, but why it does so or what the power in the serum is which brings it about, we do not know. This work with sacs shows clearly that simple neutralization of the poison (antitoxic power) avails little if the source of toxin remains; that

TABLE 8.

AFTER THE INTRODUCTION OF THE SAC THE GUINEA-PIGS RECEIVED DAILY INJECTIONS OF ANTITOXIN.

Four units daily saved the pig, lesser amounts did not.

SERUM		TOXIN IN SAC		INJECTION	DEATH	INTERVAL IN DAYS
℞ No.	Dose	℞ No.	Dose	Day	Day	
013069 Inj. daily	0.1 unit 0.1 unit	05656 up	0.15 to	2–23–10 3– 7–10	3– 8–10	13
013069 Inj. daily	0.25 unit 0.25 unit	05656 up	0.15 to	2–23–10 3– 7–10	3– 8–10	13
013069 Inj. daily	0.5 unit 0.5 unit	05656 up	0.15 to	2–23–10 3– 6–10	3– 7–10	12
013069 Inj. daily	1.0 unit 1.0 unit	05656 up	0.15 to	2–23–10 3– 8–10	3– 9–10	14
013069 Inj. daily	2 units 2 units	05656 up	0.15 to	2–23–10 3– 9–10	3–10–10	15
013069 Inj. daily	4 units 4 units	05656 up	0.15 to	2–23–10 3–13–10	Released 3–30–10 in good condition.	

an equally essential factor in cure is the stopping of the supply of toxin. We can test the neutralizing or antitoxic value of antidiphtheric sera accurately, but for this other equally important function—its action on the membrane—we have no test at present.

Prophylactic Tests.—The destruction of antitoxin *in vivo* plays so important a rôle that further tests were made to determine the rate of destruction from the standpoint of prophylaxis. The test was so arranged that a series of guinea-pigs all received the same dose of antitoxin, but on different days. After all had been injected with antitoxin they were all at one sitting injected with toxin to determine which were still protected by the serum. The injections of serum had been so arranged that the times intervening between the injection of serum and of toxin formed a regular series as 14, 12, 10, 8, 6, 4, 2 days.

TABLE 9.

BOTH TOXIN AND ANTITOXIN ADMINISTERED IN COLLODION SACS.

The guinea-pigs receiving large doses of antitoxin were not injured by the toxin.

GUINEA-PIG	SERUM IN TWO SACS		TOXIN IN SAC		INTRODUCTION OF SACS	DEATH	
Weight	℞ No.	Dose in Units	℞ No.	Dose	Day	Day	Hour
400	013069	2700	05656	0.15	4–21–10	Released 5–10–10 in good condition.	
"	"	540	"	"	"	Released 5–10–10 in good condition.	
"	"	108	"	"	"	4–29–10	

Table 10 shows the results with two series, one receiving 10 units each and the other 5 units each. If we neglect the death of one pig after 7½ days, the results show that the destruction of antitoxin is proportional to the amount of serum injected and the time. Those animals receiving 10 units were protected for four days and those receiving five units for two days.

The antitoxic power of blood serum seems to be intimately associated with certain of the globulins, and it is probable that the guinea-pig's power of destroying the antitoxin is limited to his power of destroying these foreign protein substances. Hence one would expect that the more protein the antitoxin was combined with the longer it would escape destruction.

An attempt was made to test this by using high potency serum (900 units per c.c.) in one series and low potency serum (250 units per c.c.) in the other. The results are given in Tables 10 and 11. Ten units of the high potency serum protected for 6 days and 5 units for 2 days. In both cases the low potency serum gave longer protection than the high potency serum, 6 and 2 as compared with 4 and 1.

An attempt was made to determine whether the admixture of normal horse serum or normal guinea-pig serum would prolong the time the antitoxin would protect the animal, but the difficulty in obtaining horse serum free of antitoxic power, and lack of time and opportunity to follow it up prevented getting definite results.

TABLE 10.

TO SHOW LENGTH OF TIME ANTITOXIN REMAINS IN THE GUINEA-PIGS, THEY WERE INJECTED IN SUCCESSION ONE OR TWO DAYS APART WITH ANTITOXIN. AT THE END OF THE SERIES THEY ALL RECEIVED THE SAME DOSE OF TOXIN.

The dose of toxin would be neutralized by 0.55 unit antitoxin.

GUINEA-PIG	SERUM		TOXIN		INJECTION	INTERVAL IN DAYS	DEATH	INTERVAL IN HOURS	RELEASE
Weight	R No.	Dose	R No.	Dose	Day		Day		
250	013069	10 units	.....	....	2- 3-10	14	2-18-10	22	
"	"	"	.....	....	2- 5-10	12	"	"	
"	"	"	.....	....	2- 7-10	10	"	"	
"	"	"	.....	....	2- 9-10	8	"	26	
"	"	"	.....	....	2-11-10	6	2-20-10	72	
"	"	"	.....	....	2-13-10	4	....	..	3-15-10
"	"	"	.....	....	2-15-10	2	....	..	3-15-10
All the above	inj. with		05656	0.15	2-17-10				
250	013069	5 units	.....	....	2- 9-10	8	2-18-10	22	
"	"	"	.....	....	2-11-10	6	"	26	
"	"	"	.....	....	2-12-10	5	2-19-10	36	
"	"	"	.....	....	2-13-10	4	2-20-10	72	
"	"	"	.....	....	2-14-10	3	"	72	
"	"	"	.....	....	2-15-10	2	2-25-10	7½days	
"	"	"	.....	....	2-16-10	1	....	..	3-15-10
All the above	injected	with	05656	0.15	2-17-10				

These prophylactic tests emphasize the fact that from the time of its introduction into the system antitoxin tends to be destroyed and eliminated; that the rapidity with which it is destroyed probably depends on the ability of the animal to dispose of the foreign protein with which it is associated and hence 10 units of low potency serum will remain undestroyed and protect the animal longer than 10 units of high potency serum.

An Unexplained Phenomenon.—In this work with diphtheria toxin in a collodion sac the question arose as to the amount of toxin still left in the sac after the death of the animal. To determine whether enough was left to kill another guinea-pig the sac was removed from the dead pig and transferred to the peritoneal cavity of a second guinea-pig. To our surprise the second guinea-pig died in a shorter time than the first. The test was repeated a number of times and usually the first animal died in about 5 to 7 days, the second in 2 or 3 days, the third in 3 or 4 days, and the fourth in about the same time as the first. The experiments in detail are shown in Table 12.

TABLE 11.

TO SHOW LENGTH OF TIME LOW POTENCY SERA REMAIN IN THE GUINEA-PIG, COMPARE WITH TABLE 10.

The serum was injected subcutaneously, the toxin intraperitoneally.

GUINEA-PIG	SERUM		TOXIN		INJECTION	INTERVAL IN DAYS	DEATH	INTERVAL IN HOURS	RELEASE
Weight	Name and ℞ No.	Dose	℞ No.	Dose	Day		Day		
250	013327	10 units	.....	....	2– 3–10	14	2–18–10	22	
"	"	"	.....	....	2– 5–10	12	"	"	
"	"	"	.....	....	2– 7–10	10	"	"	
"	"	"	.....	....	2– 9–10	8	"	"	
"	"	"	.....	....	2–11–10	6			3–15–10
"	"	"	.....	....	2–13–10	4			"
"	"	"	.....	....	2–15–10	2			"
All the above	injected	with	05656	0.15	2–17–10				
250	013327	5 units	.....	....	2– 9–10	8	2–18–10	26	
"	"	"	.....	....	2–11–10	6	"	"	
"	"	"	.....	....	2–12–10	5	2–19–10	36	
"	"	"	.....	....	2–13–10	4	2–20–10	72	
"	"	"	.....	....	2–14–10	3	2–20–10	72	
"	"	"	.....	....	2–15–10	2			3–15–10
"	"	"	.....	....	2–16–10	1			3–15–10
All the above	injected	with	05656	0.15	2–17–10				

It should perhaps be stated that in every case when a sac was removed bacteriologic tests were made of the peritoneal fluid on culture media containing unheated animal protein under both aerobic and anaerobic conditions. Usually the peritoneum was sterile. The few cases in which infection was found are not included.

In considering these results there seemed but two possible explanations: (*a*) that the sac became more permeable to the toxin or (*b*) that the toxin was in some way activated. To determine whether a sac would become more permeable we took a sac and filled it with a 25 per cent solution of sodium chloride in water and suspended it in a flask containing 100 c.c. of distilled water. At intervals 1 c.c. of the water was titrated for salt with silver nitrate solution. Curve 1 shows the increase of salt content to approximate very closely to a regular curve; there is no indication of increased permeability to salt.

Again to test directly for an increased permeability of the sac to toxin we took a sac from an animal just dead and instead of transferring it to a second guinea-pig placed it in a test-tube containing 3 c.c. of sterile water and kept it in the incubator for five days.. As a control we used a sac containing the usual dose of toxin (0.15 c.c.) but one which had never been in a guinea-pig and placed that in 3 c.c. of water in the incubator. Both sacs were kept under similar conditions for the same length of time and then the water from each was injected into a guinea-pig. Neither guinea-pig showed any toxic effect. Thinking the prolonged stay in the incubator might weaken the toxin, a second control was run at the same time to determine this point in this way: one, two, three, four, and five fatal doses of toxin (.005, .010, .015, .020, .025 c.c.) were diluted each to 3 c.c. with water and kept in the incubator with the sacs for the same length of time. Each amount was then injected into

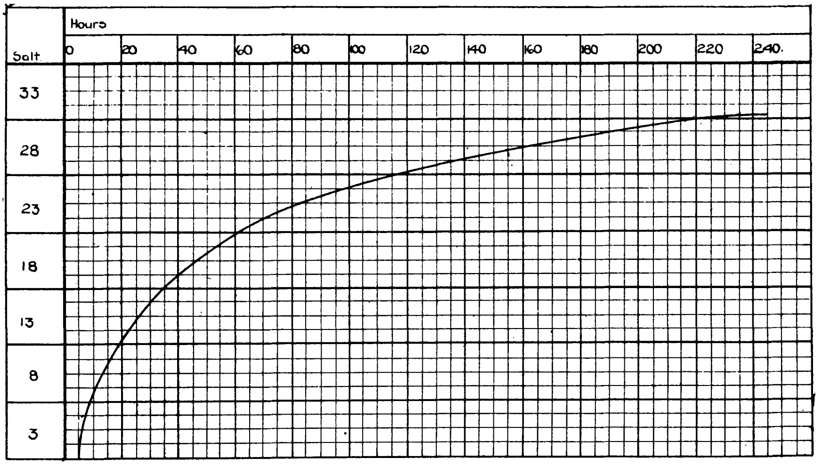

CHART 1.

a guinea-pig. All the guinea-pigs died: the one receiving one fatal dose in six days, the one receiving two fatal doses in three days, and all the rest in two days, thus showing that the toxin was not appreciably weakened by its stay in the incubator.

All attempts to obtain diffusion of toxin *in vitro* from collodion sacs have failed.

TABLE 12.

Showing the Time of Death When a Series of Guinea-Pigs Is Killed with One Collodion Sac. The second guinea-pig dies in a much shorter time than the first.

	Guinea-Pig	Toxin in Sac		Introduction of Sac	Death	Interval in Days
	Weight	℞ No.	Dose	Day	Day	
Sac 1............	400	05656	0.15	10- 1-09	10- 6-09	5
	"	Sac 1		10- 6-09	10- 8-09	2
	"	"	"	10- 8-09	10-12-09	4
	"	"	"	10-12-09	10-19-09	7
Sac 2............	400	05656	0.15	10- 6-09	10-12-09	6
	"	Sac 2		10-12-09	10-14-09	2
	"	"	"	10-14-09	10-18-09	4
Sac 3............	400	05656	0.15	10-19-09	10-24-09	5
	"	Sac 3		10-25-09	10-28-09	3
Sac 4............	400	05656	0.15	10-28-09	11- 3-09	6
	"	Sac 4		11- 3-09	11- 6-09	3
	"	"	"	11- 6-09	11- 9-09	3
Sac 5............	400	05656	0.15	11-18-09	11-24-09	6
	"	Sac 5		11-24-09	11-26-09	2
Sac 6............	400	05656	0.15	12-21-09	12-26-09	5
	"	Sac 6		12-27-09	12-29-09	2
	"	"	"	12-29-09	1- 2-10	4
Sac 7............	400	05656	0.15	12-21-09	12-27-09	6
	"	Sac 7		12-27-09	12-30-09	3
Sac 8............	400	05656	0.15	1-25-10	2- 1-10	7
	"	Sac 8		2- 1-10	2- 3-10	2
Sac 9............	400	05656	0.15	1-25-10	1-30-10	5
	"	Sac 9		1-31-10	2- 2-10	2
Sac 10...........	400	05656	0.15	2- 7-10	2-15-10	8
	"	Sac 10		2-15-10	2-18-10	3

To determine how long after its introduction into the first pig the sac acquires the power of killing the second pig quickly, sacs were removed from the animals at varying intervals and placed in fresh pigs. The animals from which the sacs were to be removed were anesthetized, the wound opened, the sac removed carefully, and the wound again closed. In all cases they made good recoveries and seemed none the worse for the operations or the toxin. The sac removed 24 hours after its introduction killed a second pig in four days, one 36 hours after in three days, and one 48 hours after in three days also. Here we have a sac remaining 48 hours in a pig and doing him so little damage that he could stand the toxin, two operations, and the trauma and shock incident to removal of the sac (the omentum tends to adhere to the sealed end of the sac so that it is difficult to remove the sac without trauma to the omentum); yet this same sac when transferred to a second pig kills it in 72 hours. The fact that these pigs die of toxin poisoning was in all cases controlled by the postmortem appearances, the absence of infection, and the uniformity of results.

Again we made a test to determine how much toxin was left in a sac after the first pig was dead. The entire contents of a sac from a guinea-pig that died in 6 days was divided into portions and injected into guinea-pigs in the following amounts: $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$ of the contents of the sac. All died except the one receiving the $\frac{1}{32}$ dose, and from the fact that the pig receiving the $\frac{1}{16}$ dose died in three days, it is probable that there were at least 20 M.L.D. left in the sac, or about half the amount put in (37½ to 40). Yet this sac with only half the original amount of toxin would kill a second guinea-pig in less than half the time required to kill the first pig.

SUMMARY.

In this work it has been shown:

1. That a guinea-pig may be killed by introducing a collodion sac containing diphtheria toxin into its peritoneal cavity; the slow absorption of toxin from the sac corresponding quite closely to the absorption of toxin from the false membrane in a case of diphtheria.

2. That to save the life of a guinea-pig by the administration of antitoxin after the introduction of a sac, requires an enormous dose or the long continued administration of moderate doses. The reason for this seems to be that the antitoxin administered subcutaneously, being a foreign protein, is subjected to rapid destruction by the tissues, while the toxin protected from such destruction by the sac continues to diffuse out and kills the animal after the protective power of the antitoxin has disappeared. The essential difference between these sac tests and the administration of antitoxin in diphtheria appears to be that in these tests the antitoxin has no

influence on the supply of toxin, while in diphtheria the administration of antitoxin is followed by destruction of the membrane, and consequently the supply of toxin ceases and the patient recovers. This power of antidiphtheric serum to destroy the membrane is probably quite as important as its antitoxic action, but unfortunately we have no means of measuring it.

3. That when used as a prophylactic, low potency sera protect for a longer time than those of high potency. Antitoxin protects until it is destroyed by the tissues. The more proteid it is combined with the longer will its destruction be delayed.

4. That diphtheria toxin in a collodion sac undergoes some change by which the sac altho containing less toxin than in the beginning is able to kill a second guinea-pig in half the time required to kill the first. This difference may be analogous to the short incubation period of tetanus toxin from the blood of an animal compared with the incubation period of tetanus toxin produced by the growth of the germs *in vitro.* It may also account for certain very rapidly fatal cases of diphtheria.